Video Article

Modeling Amyloid-β42 Toxicity and Neurodegeneration in Adult Zebrafish Brain

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Abstract

Alzheimer's disease (AD) is a debilitating neurodegenerative disease in which accumulation of toxic amyloid-β42 (Aβ42) peptides leads to synaptic degeneration, inflammation, neuronal death, and learning deficits. Humans cannot regenerate lost neurons in the case of AD in part due to impaired proliferative capacity of the neural stem/progenitor cells (NSPCs) and reduced neurogenesis. Therefore, efficient regenerative therapies should also enhance the proliferation and neurogenic capacity of NSPCs. Zebrafish (*Danio rerio*) is a regenerative organism, and we can learn the basic molecular programs with which we could design therapeutic approaches to tackle AD. For this reason, the generation of an AD-like model in zebrafish was necessary. Using our methodology, we can introduce synthetic derivatives of Aβ42 peptide with tissue penetrating capability into the adult zebrafish brain, and analyze the disease pathology and the regenerative response. The advantage over the existing methods or animal models is that zebrafish can teach us how a vertebrate brain can naturally regenerate, and thus help us to treat human neurodegenerative diseases better by targeting endogenous NSPCs. Therefore, the amyloid-toxicity model established in the adult zebrafish brain may open new avenues for research in the field of neuroscience and clinical medicine. Additionally, the simple execution of this method allows for cost-effective and efficient experimental assessment. This manuscript describes the synthesis and injection of Aβ42 peptides into zebrafish brain.

Video Link

The video component of this article can be found at https://www.jove.com/video/56014/

Introduction

AD is a chronic progressive disease characterized by the loss of neurons and synapses in the cerebral cortex[1,2,3,4,5]. The classical neuropathological hallmarks of AD are the deposition of amyloid peptides and formation of the neurofibrillary tangles (NFTs)[6]. Senile plaques, also known as amyloid plaques, are composed of amyloid-β (Aβ) peptides that form β-pleated structures in the brain parenchyma[5]. The accumulation of Aβ42 in AD patients has an early and critical role in disease progression. AD triggers a cascade of events leading to synaptic dysfunction, impaired plasticity, and neuronal loss[7,8,9,10].

The adult brain of teleost zebrafish serves as an excellent model to study the regulation of stem cell plasticity[11,12,13,14,15,16,17,18,19,20] and various diseases in the central nervous system (CNS), including AD[21,22,23,24]. Owing to a vast array of available experimental methods[19,20,25,26,27,28,29,30,31], these studies are informative and feasible. Zebrafish can replenish the CNS[13,15,32,33,34,35,36,37,38], in part by using molecular programs activated after neuronal loss[19,39,40,41,42,43,44]. Therefore, establishing a neurodegenerative disease model in zebrafish can help address novel questions regarding regenerative ability and stem cell biology in vertebrate brains.

Recently, we developed an amyloid toxicity model in adult zebrafish brain by injecting synthetic Aβ42 peptides (**Table 1**)[39]. This injection caused neurodegeneration phenotypes reminiscent of human brain pathology (*e.g.*, cell death, microglial activation, synaptic degeneration, and memory deficits), indicating that zebrafish can be used for eliciting neurodegeneration in zebrafish brain, Aβ42 peptides can be detected with immunohistochemical stainings, and molecular mechanisms of regeneration in adult zebrafish CNS can be identified[39]. In this protocol, we demonstrate the injection of synthetic amyloid peptides into the zebrafish brain using a cerebroventricular injection (CVMI) method[27,39,45,46] to mimic amyloid deposition (**Figure 1**). CVMI provides a novel way of delivering the peptides, which aggregate upon injection as β-sheet structures and exert toxicity. The peptides are distributed evenly throughout the brain, targeting the ventricular area along the entire rostro-caudal axis[45]. Additionally, this method allows for analyzing the morphological and molecular response of the NSPCs in adult zebrafish brain following amyloid inclusions. Such studies will provide us an insight for successful brain repair in mammals. Our method can be used to understand the necessary molecular mechanism of a successful regeneration response after AD-like symptoms to induce replenishment of lost neurons and functional recovery.

Protocol

This protocol is a standard procedure suggested by the EU guidelines (2010/63) and the European Society for Fish Models in Biology and Medicine (EuFishBioMed) in Karlsruhe Insitute of Technology (KIT). All methods described after here have been approved by the ethics commission (Landesdirektion Dresden; document number TVV-52/2015).

1. Preparation of Aβ42 Peptide

1. Synthesize peptides (see **Table 1**) using the standard 9-fluorenylmethoxycarbonyl (Fmoc) chemistry with 2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronoiumhexafluorphosphate (HBTU) as the coupling reagent on an automated solid-phase peptide synthesizer[52]. The scale of the synthesis was 100 µmol.
2. Load the automated peptide synthesizer with 500 mg of the Fmoc-protected resin as the solid phase (loading capacity of 0.2 mmol/g). Load the dissolved Fmoc-protected amino acids at a concentration 0.5 M in the volume required and as calculated for the respective synthesizer.
   NOTE: The calculations are made to enable the coupling of each Fmoc-protected amino acid twice with 5 times excess of each building block to the resin[47].
3. Dissolve the required reagents for peptide synthesis in dimethlyformamide (DMF). For example, prepare the activator, HBTU, at a concentration of 0.48 M, 45% v/v N-Methylmorpholine (NMM) (the base), and 5% v/v Acetic Anhydride (the capping mixture) to cap the non-reacted amino groups.
4. Cleave all the peptides from the resin by continuously mixing the solid support using an agitator in a freshly prepared cleavage mixture consisting of Trifluoroacetic acid (TFA): Triisopropylsilane(TIS): water: Dithiothreitol (DTT) at 90 (v/v): 5 (v/v): 2.5 (v/v): 2.5 (m/v), for 4 h. Use 10 mL for the synthesis scale of 100 µmol.
5. Precipitate the cleaved product by adding the cleavage mixture to 100 mL ice-cold diethyl ether. Pass through a filtration unit containing a Polytetrafluoroethylene (PTFE) filter with a pore size of 0.45 µm and wash with 20 mL ice-cold diethyl ether.
6. Collect the filtered peptide from the filter paper and dissolve 100 mg of precipitated peptide in 5 mL distilled deionized water: acetonitrile at 1:1.
7. **Purify via reverse-phase high-pressure liquid chromatography (HPLC) on a semi-preparative HPLC equipped with a porous polystyrene divinylbenzene column of bead size 10 µm.**
   1. Pre-heat the column and maintain at 50 °C using a column heating device. Collect all the major fractions using an automated fraction collector by applying a gradient from 5% to 100% solvent B over 25 min at 4 mL/min.
      NOTE: Solvent A is 0.1% TFA in water and solvent B is 0.1% TFA in acetonitrile[52].
   2. Monitor the chromatogram at 220 nm, collect the appropriate peaks, and analyze using the LC-MS.
8. Confirm the purity by analytical reverse phase ultra-high Pressure Liquid Chromatography (UPLC) at 220 nm by monitoring with a UV Detector while passing the sample through an analytical C18 column (bead size 1.7 µm). Confirm the peptide product by mass spectrometry.
   NOTE: The UPLC is coupled to an electrospray ionization mass spectrometry (ESI-MS) and Tandem Quadrupole Detector.
9. Lyophilize the correct fractions of the desired peptide in a round bottom flask, by applying a vacuum of 0.052 mbar, to a fluffy powder. Couple the vacuum pump to a freezing unit maintained at -78 °C. Store at -80 °C, indefinitely.
10. Use the lyophilized peptide to prepare a stock solution of 1 mM in a mixture of acetonitrile: DMF: analytical grade water at 1:1:1 for the experiments. This solution can be stored for at least 6 months, as the peptides do not aggregate in this solution.

2. Preparation of the Injection Mixture

1. Prepare phosphate-buffered saline (PBS) (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4, pH 7.4) for diluting the lyophilized peptides.
2. Dissolve the peptide to a final concentration of 20 µM in PBS. Prepare this solution fresh. Mix well and store on ice until the injection. Do not exceed 30 min to prevent aggregation in solution.

3. Anesthesia

1. Prepare the stock solution of the anesthetics, 0.1% ethyl-m-aminobenzoate methanesulphonate (MESAB), in regular fish water from the circulating system. Prepare the anaesthetization solution to a final concentration of 0.0025% (v/v).
2. Remove the desired number of fish from their tanks into a transport container with 5 L of system water.
3. Half-fill a plastic Petri dish (90 mm) with 40 mL anesthetics. Use this dish for injections.
4. Incubate the fish in the anesthetics until the opercular movement has ceased.

4. Cerebroventricular Microinjection

1. **Preparation of injection apparatus**
   1. Prepare the glass injection capillaries using a needle puller with the following parameters: heating cycle, 537; pulling cycle, 250; velocity, 1.5 s; time, 80 ms.
   2. Bring the pressure setting on the pressure source to 25 psi.
   3. Set the microinjector parameters to the following: hold pressure 20 psi; eject pressure 10 psi; period value 2.5; gating value 100 ms.
   4. Load the glass capillary with the injection solution. Insert the glass capillary into the microinjection holder. Adjust the injection angle to 45°.
      NOTE: More detailed protocols can be found as described in references[27,46].

2. Place one fish into a new Petri dish filled with anaesthetization solution (as in step 3.3).
3. Hold the fish with the forceps and orient for injection.
4. Generate a slit using the tip of a 30 G needle in the skull over the optic tectum where the two lateral plates meet. Do not insert the tip into the brain tissue, this would cause bleeding and damage. See references[27,45,46] for additional details.
5. **Insert the glass capillary into the slit.**
    1. Use only the tip of the needle and do not penetrate more than 1 mm through the skull.Keep holding the fish and insert the tip of the glass capillary through the incision site.
    2. Orient the tip of the glass capillary towards the telencephalon at a 45° angle. Inject 1 µL of the solution. The liquid disperses immediately after injection.

5. Recovery

1. Place the fish back to a transport container until it recovers. Connect the container to the regularly circulating fish water to ensure optimum water quality.
   NOTE: The recovery should normally take 1 min. If it takes longer, the fish must be kept in the anesthetics for a shorter time (this needs to be optimized by the experimenter).

6. Tissue Preparation and Sectioning

1. Wait for a desired period of time before sacrificing the fish.
   NOTE: This depends on the experimental question. Amyloid deposition can be seen as early as 1 day after injection.
2. Sacrifice the fish using the appropriate method depending on the ethical regulations (*e.g.*, treat the fish with 0.1 M MESAB).
3. Cut open the skull above the optic tectum using pointed forcep on the dorsal side, and dissect the head just behind the pectoral fin using a scalpel.
4. Fix the heads using 2% paraformaldehyde (PFA) overnight at 4 °C. Use 3 mL of PFA per head in a plastic tube with a screw lid.
   CAUTION: Wear the appropriate personal protective equipment when handling PFA.
5. For cryoprotection and decalcification, wash the heads thrice in 0.1M Phosphate Buffer (pH 7.4), then transfer them into 20% sucrose/20% ethylenediaminetetraacetic (EDTA) solution and incubate overnight at 4 °C.
6. To embed the tissue into sectioning resin, freeze the heads in 7.5% gelatin/20% sucrose solution in plastic histology molds on dry ice (approximately 3-5 min to freeze a block). Store the samples at -80 °C or continue to the next step (cryosectioning).
7. Section the heads into 12 µm thick cryosections using a cryostat as described[28,42]. Transfer the cryosections directly onto glass slides and store at -20 °C for long-term usage.

7. Immunohistochemical Staining and Microscopy

NOTE: Perform all incubation steps in a humidified chamber. And, all the washing steps are for 10 min each.

1. Dry the sections for 30 min at room temperature. After thawing, wash the sections twice in PBS and once in PBSTx (PBS with 0.03% Triton-X-100).
2. Incubate with the primary antibody (anti-Aβ42 antibody; 1:500 dilution in PBSTx) at 4 °C overnight. Following day, wash once in PBS and twice in PBSTx.
3. Incubate the sections with the secondary antibody (fluorescence-coupled detection, 1:500 dilution) along with DAPI (1 µg/mL) in PBSTx for 2 h at room temperature.
4. Wash three times in PBSTx. After the final washing, mount the slides with a coverslip using 100 µL 70% glycerol.
5. Acquire fluorescent images using a confocal microscope (for example with 20X PL APO N.A. 0.7 objective with 488 excitation range and a standard green filter; **Figure 2**)[39].

Representative Results

HPLC was used to purify the synthesized peptide and mass spectrometry has been used to characterize the purified amyloid β peptides. The HPLC column was heated to 50 °C to improve the separation of the Aβ peptides and all the fractions were collected. To identify the correctly synthesized peptide, mass spectroscopy analysis was performed for all fractions. The UPLC chromatogram shows the purity of the compound. The HPLC fraction that yielded one peak on the UPLC (*i.e.*, the correct mass to charge ratio of the required amyloid β peptide) was further processed for experiments (**Figure 2**).

The functionality of the peptides in forming aggregates can be assessed using various spectroscopic methods[39,48,49], and also by incubating the peptides in PBS at room temperature for more than 1 h, which will yield aggregates. In this case, precipitates and large aggregates should be seen in solution.

After cerebroventricular microinjection, the peptides aggregate in the brain and form amyloid depositions (**Figure 3**). These aggregates are mostly seen as intracellular depositions, but also around the blood vessels. Such aggregations are indications of a successful accumulation of amyloid peptides in the tissue.

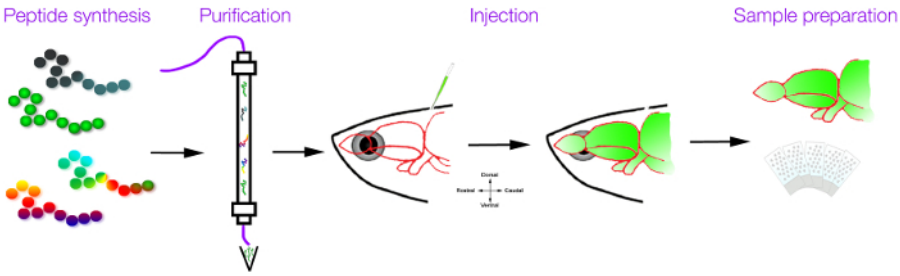

Figure 1: Schematic representation of the peptide synthesis, purification, injection, and analyses. Please click here to view a larger version of this figure.

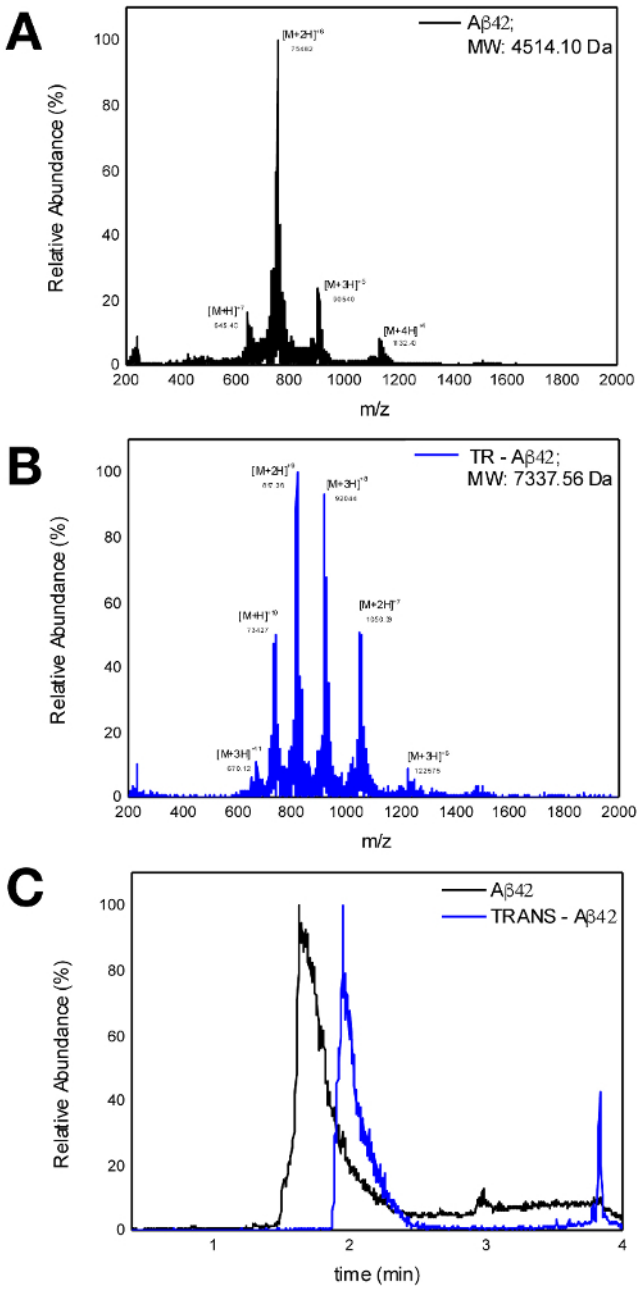

Figure 2: Characterization of native amyloid-β42 peptide. Mass spectrometry characterization of native amyloid-β42 peptide (**A**) and the CPP-tagged amyloid-β42 peptide (TR-Aβ42; **B**). (**C**) The chromatograph of the purified, native, and TR-Aβ42 peptide on the Ultra-high Pressure Liquid Chromatography (UPLC). X-axes denote the mass-to-charge ratio (m/z) (**A**, **B**) and the elution time in minutes (**C**). Please click here to view a larger version of this figure.

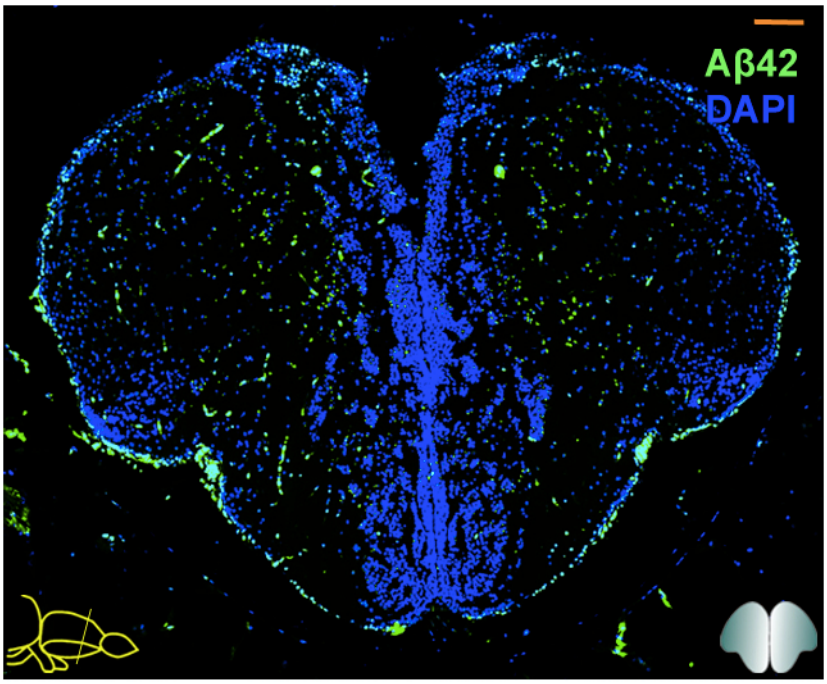

Figure 3: Immunohistochemical staining of amyloid deposition in an adult zebrafish brain section 1 day after injection. Aβ42 is in green, and nuclei are in blue. Detection of green clusters indicates efficient Aβ42 aggregation. Scale bars = 100 µm. This figure has been modified from reference[39].

Peptide	Peptide Sequence	MW (g/mol)
Aβ42	DAEFRHDSGYEVHHQKLVFFAEDVGSNKGAIIGLMVGGVVIA	4514.1
TR-Aβ42	GWTLNSAGYLLGKINLKALAALAKKILDAEFRHDSGYEVHHQKLVFFAEDVGSNKGAIIGLMVGGVVIA	5819.8

Table 1: Aβ42 and TR-Aβ42 peptide sequences.

Discussion

The amyloid peptides can be modified to include sequence variations or various tags. For instance, a scrambled amyloid peptide can be generated, and the peptides can be labeled with fluorescent tags at the N-terminus of the peptide end or tagged with carrier peptides[39]. Similarly, in this protocol, the carrier peptide is the cell-penetrating peptide TR because of its effectiveness to transport cargo deep into the brain tissue[39]. Additionally, our method allows for injection and analyses of various peptides that can cause toxic aggregations[50,51]. Therefore, our system offers a versatile method for manual injection of toxic proteins into zebrafish brain and analyzing the cellular effects of such toxicity.

Amyloid peptides aggregate quickly, and therefore, the stocks must be kept in the water-DMF-acetonitrile solution, and should be mixed with PBS only 0.5 h before the injection. If there are large aggregates in the solution, the injection solution should be prepared only 10 min before the injection. Injection efficiency is an important parameter for yielding consistent results. Please refer to our previous JoVE paper[46] for performing a good and consistent injection. If no aggregation is seen after injection, the injection method must be optimized.

Our method is limited in terms of the size of the molecules to be injected. We previously showed that the plasmids or short oligomers can be taken up efficiently by the ventricular cells[45,46], however, for efficient delivery into deep brain tissues, large molecules (*e.g.*, antibodies, large proteins) cannot be used. Additionally, lipophilic molecules may not easily penetrate the deep tissues because such molecules will be sequestered by the first few layers of ventricular cells.

The generation of mouse models of neurodegeneration is time-consuming and maintenance of these models is quite expensive. Our injection method is a rapid model for toxic amyloid deposition, and neurodegeneration. Additionally, the zebrafish has a superior regenerative ability compared to mammalian models, and investigations focusing on how vertebrate brains could mount a regeneration response after neurodegeneration will surely benefit from rapid assay systems in zebrafish.

The quality of the synthesized peptide and its purity are important for the success of the experiment. To ensure this quality, synthesized peptides must be thoroughly characterized using liquid chromatography and mass spectroscopy. Additionally, circular dichroism and aggregation studies as described[39] are suggested.

Injection into the fish brain is a critical step to ensure a consistent aggregation and pathological outcome. A longitudinal study that analyzes the aggregation and clearance dynamics of the amyloid peptides can be performed, if desired. We use an amyloid peptide coupled to a carrier peptide to ensure equal distribution and penetration into the brain tissue. Uncoupled amyloid peptides also give similar results but the timeline of distribution and aggregation is different[39]. The experimenter can choose the desired version of amyloid peptides depending on the aim.

There is great potential for use of our method in combination with other manipulation studies. CVMI of amyloid peptides can be combined with drug treatments or injection of other compounds to test the synergistic effects of various molecules in a disease condition. Ultimately, our rapid and novel model can also be used for small-scale drug-screening approaches in an easy laboratory setting.

Our manual microinjection method can be efficiently used to inject amyloid peptides into the adult zebrafish brain to mimic amyloid deposition. Amyloid depositions in the brain elicit cytotoxic effect and results in AD pathology, hence displaying an acute neurodegenerative condition. The future outlook for this method would be to utilize it in an acute degenerative model to study the neuropathology in zebrafish brain and the regenerative response thereof. Such understanding will provide an important platform to design new therapeutic strategies.

Disclosures

The authors have nothing to disclose

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